Schizophrenia Research 212 (2019) 107–112

Contents lists available at ScienceDirect

Schizophrenia Research

journal homepage: www.elsevier.com/locate/schres

Targeted lipidomics and metabolomics evaluations of cortical neuronal stress in schizophrenia

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ARTICLE INFO

Article history:
Received 3 June 2019
Received in revised form
2 August 2019
Accepted 3 August 2019
Available online 18 August 2019

Keywords:
Schizophrenia
Sphingolipids
Plasmalogens
N-Acylphosphatidylserines
N-Acylphosphatidylethanolamines
N-Acetylaspartatylglutamate
N-Acylethanolamines
N-Acyltaurines
N-Acetylneuraminic acid

ABSTRACT

Background: Cortical neuronal dysfunction has been proposed to underlie the psychopathology and cognitive dysfunction of schizophrenia. Previously we have reported altered sphingolipid and *N*-acylphosphatidylserine (NAPS) metabolism in the frontal cortex in schizophrenia. We continue to expand these investigations to define the biochemical basis for these critical neuropathologies.
Methods: We undertook a targeted high resolution mass spectrometric analysis to validate our previous reports of elevated sphingolipids and NAPS in the frontal cortex of a new cohort of schizophrenia subjects. Furthermore we expanded these analyses to include ceramides, *N*-acylphosphatidylethanolamines (NAPE), and *N*-acylethanolamines (NAE). In the same tissue samples we examined *N*-acetylaspartylglutamate (NAAG), a modulator of excitatory amino acid transmission, hypothesized to be involved in the pathology of schizophrenia.
Results: We repeated our observations of elevated sulfatides in the frontal cortex in schizophrenia. An in-depth analysis of other sphingolipids revealed decrements in ceramide levels and increased levels of lactosylceramides. NAPS also were found to be augmented in schizophrenia as we previously reported. In addition, levels of NAPES, established biomarkers of neuronal stress, were elevated while their metabolites, NAEs were decreased. With regard to excitatory amino acid neurotransmission, NAAG levels were decreased by 50% while the metabolic precursor, *N*-acetylaspartate was unaltered.
Conclusions: Our data support the concept of cortical neuronal dysfunction in schizophrenia as indicated by altered metabolism of structural sphingolipids and NAAG, a modulator of excitatory amino acid neurotransmission.

1. Introduction

The pathophysiology of schizophrenia is extremely complicated such that there currently is no integrative hypothesis that can explain the clinical triad of negative symptoms, positive symptoms, and cognitive dysfunction in this CNS disorder. The most compelling data, at this time, support excessive dopaminergic neurotransmission as the basis of psychotic symptoms in schizophrenia patients (Tamminga et al., 1995; Howes et al., 2015). Neither the dopamine hypothesis (Howes et al., 2015) nor the glutamate hypothesis (Uno and Coyle, 2019) have provided compelling evidence regarding the role(s) of these neurotransmitters in the genesis of negative symptoms and/or cognitive dysfunction in schizophrenia.

One approach to this dilemma has involved extensive brain imaging studies. Such tractography analyses have concluded that schizophrenia is characterized by anatomical/functional dysconnectivity as indicated by increased functional anisotropy in a number of white matter tracts which correlates with cognitive dysfunction in schizophrenia patients (Faria et al., 2019; Gómez-Gastiasoro et al., 2019). It is also important to note that "cognitive deficits increase the risk of impulsive aggression in schizophrenia via inefficient regulation of negative affective states" (Ahmed et al., 2018).

While there has been an extensive investigation of white matter dysfunction, there have been limited approaches to define biomarkers of neuronal stress in schizophrenia. We have previously reported elevated levels of *N*-acylphosphatidyserines (NAPS), putative biomarkers of neuronal stress, in the frontal cortex in schizophrenia (Wood, 2014; Wood and Holderman, 2015). We therefore undertook a validation of these observations in a third cohort of post-mortem frontal cortex tissues and expanded our study to investigate *N*-acylphosphatidylethanolamines (NAPEs), which are well established biomarkers of neuronal stress in models of hypoxia/ischemia (Hansen et al., 2000; Kilaru et al., 2011; Janfelt et al., 2012; Luptakova et al., 2018) and models of excitotoxicity (Hansen et al., 1997; Hansen et al., 2001; Guan et al., 2006). The potential value of such analyses is further supported by reports of

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https://doi.org/10.1016/j.schres.2019.08.003
0920-9964/

polymorphisms in NAPE-phospholipase D (NAPE-PLD; EC 3.1.4.54) and fatty acid amide hydrolase (FAAH; EC 3.5.1.99) in schizophrenia (Costa et al., 2013; Si et al., 2018). Alterations in NAPE-PLD, which cleaves NAEs (endocannabinoids) from NAPEs at sn-3, and in FAAH which regulates the levels of NAEs, are likely to contribute to decreased levels of NAEs in the cortex in schizophrenia (Muguruza et al., 2013).

In addition to the targeted analyses of NAPS, NAPEs and NAEs, we undertook, in the same cortical tissues: i) a validation study of sulfatides, which we have previously reported to be augmented in the cortex in schizophrenia (Wood et al., 2014; Wood and Holderman, 2015); ii) an in-depth analysis of ceramides, sulfatide precursors, in the frontal cortex; and iii) a targeted analysis of NAAG, a modulator of glutamate release (Jessen et al., 2013; Lieberburg et al., 2016; Uno and Coyle, 2019).

2. Materials and methods

2.1. Patient brain samples

Frontal cortex brain samples (BA 10; 10 controls and 10 schizophrenia) were provided by the NIH Neurobiobanks. Schizophrenia patients were diagnosed based on the Structural Interview for Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV), with all patients receiving antipsychotic medication. The demographics of the donors are presented in Table 1. The groups were well balanced with regard to both gender (controls = 6 M and 4 F; schizophrenia = 5 M and 5 F) and age (mean ± SD; controls = 42.6 ± 10.1 and schizophrenia = 38.7 ± 8.2 years).

2.2. High resolution mass spectrometry

For lipid analyses, frontal cortex tissue was processed as described previously (Wood, 2014; Wood et al., 2014; Wood and Holderman, 2015). Briefly, 400 to 600 mg of tissue were sonicated in 1 mL of methanol, containing stable isotope internal standards, and 1 mL of distilled water. Next, 2 mL of *tert*-butylmethylether were added and the samples shaken at room temperature for 30 min prior to centrifugation at 4000 ×*g* for 30 min at room temperature. One mL of the upper organic layer containing the lipids was transferred to a 96 deep well plate and the samples dried by vacuum centrifugation. Flow infusion analysis (FIA, 12 μL/min) was performed utilizing high-resolution (140,000 at 200 amu; 0.4 to 3 ppm mass error) data acquisition with an orbitrap mass spectrometer (Thermo Q Exactive), as reported previously (Wood, 2017). However the infusion solvent was changed to 2-propanol:methanol:dichloromethane (8:4:4) + 5 mM ammonium chloride. With this solvent, dominant $[M + Cl]^-$ anions of ceramides, hydroxyceramides, phytoceramides, hexosylceramides, lactosylceramides, and ceramide phosphoethanolamines were generated with electrospray ionization (ESI). These chloride adducts provided a 10- to 50-fold increase in sensitivity over the weaker $[M + H]^+$, $[M\text{-}H]^-$, and $[M + HCOO]^-$ ions observed with ammonium acetate as the solvent additive (Wood, 2017). All lipids were quantitated based on accurate masses obtained from the Lipid Maps database (lipidmaps.org) and identities validated by tandem mass spectrometry with a parent ion isolation window of 0.4 amu and the product ions monitored with high resolution (<2 ppm mass error).

For the analysis of NAAG, tissues were extracted with acidified acetonitrile/methanol as described previously (Smith et al., 2011). Briefly, 400 to 600 mg of tissue were sonicated in 1 mL of cold methanol: acetonitrile (200:800) containing 2.5 mL of formic acid, prior to centrifugation at 30,000 ×*g* and 4 °C for 30 min. 800 μL of the extracts were dried by vacuum centrifugation and dissolved in acetonitrile:methanol (1:1) for FIA (12 μL/min). The molecular anion for *N*-acetylneuraminic acid (308.0987) and the product anions of *N*-acetylaspartate (NAA, 174.0407 → 88.0404), *N*-acetylglutamate (NAG, 188.0564 → 102.0560), and NAAG (303.0833 → 128.0353) were monitored. Parent ions were selected with a 0.4 amu window and product ions were monitored with high resolution (0.4 to 3 ppm mass error). $[^2H_4]$*N*-acetyl-alanine and $[^2H_3]$*N*-acetyl-methionine were utilized as internal standards.

2.3. Data analyses

Data are presented as relative levels (ratio of the peak area of the endogenous lipid/metabolite to the peak area of an appropriate internal standard), corrected for tissue protein, in bar graphs ± SEM. Data were analyzed in Excel (Microsoft) utilizing the Student's *t*-test, after testing for homogeneity of variance (F-test).

3. Results

3.1. Sphingolipids

As in our previous studies (Wood et al., 2014; Wood and Holderman, 2015), sulfatides were significantly elevated in the frontal cortex samples from schizophrenia patients (Fig. 1). To monitor sphingolipid precursors and metabolites of sulfatides (Fig. 2), we performed a targeted analysis of ceramides utilizing our new assay

Table 1
Demographics of the donors for the frontal cortex samples.

Diagnosis	Age	Gender	Race	PMI (Hr.)
Schizophrenia controls	42	M	AA	25
	64	F	AA	7
	36	M	C	23
	31	M	C	26
	49	M	C	26
	46	M	C	21.7
	41	F	C	20.8
	50	F	C	13.7
	35	M	C	25.7
	32	F	C	12.3
	43	M	AA	21
	44	F	AA	27
	30	M	AA	20
	49	F	AA	24
	30	F	AA	23
	43	M	C	21.4
	47	M	C	12.5
	35	F	C	9.3
	41	F	C	11
	25	M	C	33.8

AA, African American; C, Caucasian.

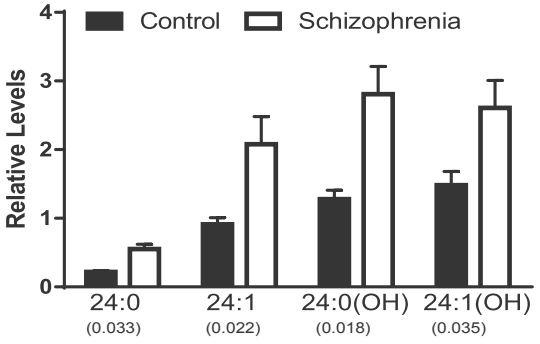

Fig. 1. Relative levels of sulfatides in the frontal cortex of post-mortem tissues from schizophrenia patients. Values in brackets are the p values.

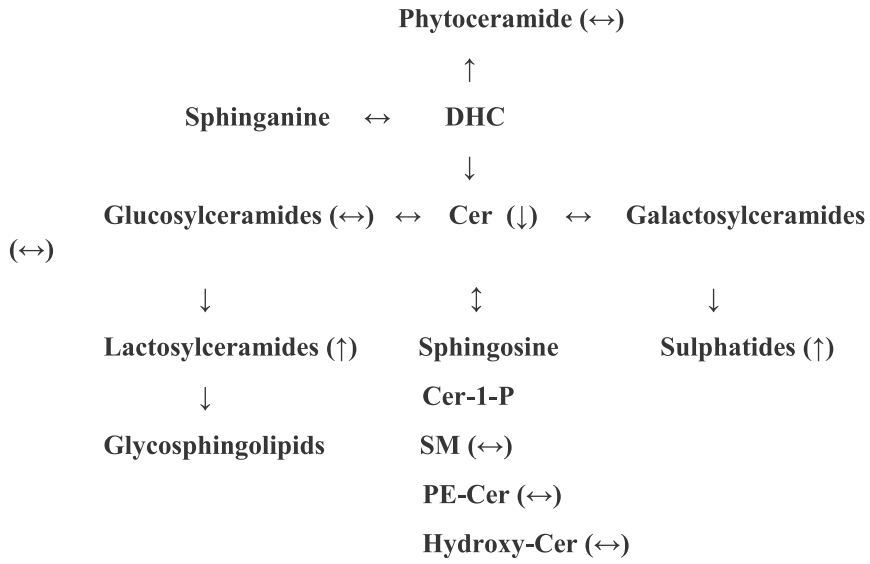

Fig. 2. Schematic overview of sphingolipid metabolism that can be accessed via high resolution mass analyses of the chloride adducts of these lipids. Cer, ceramide; DHC, dihydroceramide; P, phosphate; PE-cer, phosphoethanolamine ceramide; SM, sphingomyelin. The arrows in brackets reflect the observed alterations in the samples from patients with schizophrenia.

of the chloride adducts of these lipids. As presented in Fig. 3, ceramide levels were found to be decreased and levels of lactosylceramides elevated in the frontal cortex in schizophrenia. Levels of hydroxyceramides, phytoceramides, hexosylceramides, and phosphoethanolamine ceramides were unaltered. These data suggest that decreases in ceramide levels may be associated with augmented metabolism of these lipids to lactosylceramides and sulfatides via the glucosylceramide and galactosylceramide pathways, respectively (Fig. 2). In contrast to our prior study (Wood et al., 2014), we did not monitor increases in hexosylceramides. This may be due to our FIA analysis which does not distinguish between glucosylceramides and galactosylceramides, or be the result of a more specific assay with the chloride adduct of these lipids.

3.2. NAPS and NAPES

In our new set of post-mortem tissues we repeated our prior observations (Wood, 2014; Wood and Holderman, 2015) of elevated levels of NAPS in the frontal cortex in schizophrenia (Fig. 4). Since these may be biomarkers of neuronal stress, we also examined NAPEs and plasmenyl NAPEs (NAPEp), which are more established biomarkers of neuronal stress (see Introduction). These complex lipids also were augmented in the frontal cortex (Fig. 4). In contrast, NAEs, the endocannabinoid metabolites of NAPEs, were found to be decreased in parallel with the accumulation of NAPEs (Fig. 5). Also of interest, we noted a specific decrease in the relative levels of *N*-arachidonyltaurine (NAT 20:4) in schizophrenia (Fig. 5).

3.3. N-Acetyl amino acids

N-Acetylaspartate and *N*-acetylglutamate levels were unaltered in the frontal cortex in post-mortem samples from schizophrenia patients, while the neuromodulator NAAG was significantly reduced (Fig. 6). These data are consistent with a previous post-mortem analysis (Jessen et al., 2013). *N*-Acetylaspartylglutamylglutamate (Fig. 7), which is present more caudally in the nervous system (Lodder-Gadaczek et al., 2011), was not detected in our frontal cortex samples.

4. Discussion

Our data from a third cohort of post-mortem tissues validate augmented sulfatide levels in the frontal cortex in schizophrenia (Wood et al., 2014; Wood and Holderman, 2015). Sulfatides, which are synthesized by oligodendrocytes (Eckhardt, 2008), are essential components of glycosynapses that regulate axonal metabolic function and provide trophic support to axons (Boggs et al., 2010; Boggs, 2014). Accumulation of sulfatides, may contribute to the

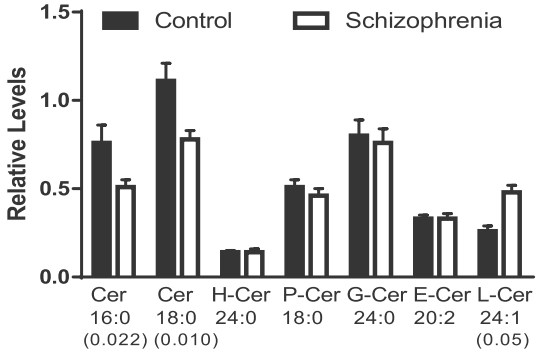

Fig. 3. Relative ceramide levels in the frontal cortex in schizophrenia. Cer, ceramide; H-Cer, hydroxyceramide; P-Cer, phytoceramide; G-Cer, hexosylceramide; E-Cer, phospoethanolamine ceramide; L-Cer, lactosylceramide. Values in brackets are the p values.

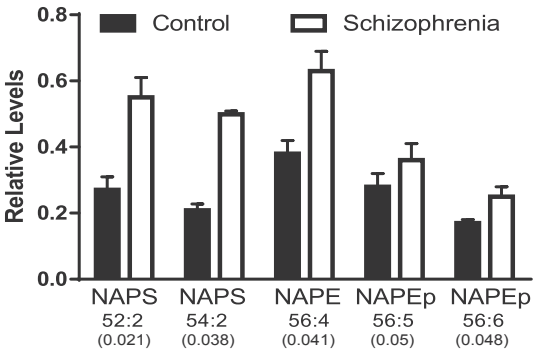

Fig. 4. Relative levels of NAPS, NAPE, and NAPEp in the frontal cortex in schizophrenia. Values in brackets are the p values.

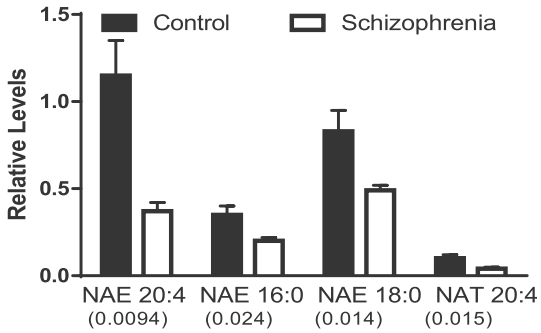

Fig. 5. Relative levels of *N*-acylethanolamines (NAE) and *N*-arachidonyltaurine (NAT 20:4) in the frontal cortex in schizophrenia. Values in brackets are the p values.

formation of less compact myelin (Uranova et al., 2011) in schizophrenia which can be monitored in vivo via analysis of fractional anisotropy (Faria et al., 2019; Gómez-Gastiasoro et al., 2019). Also of significance are the observations that sulfatide accumulation in metachromatic leukodystrophy is associated with psychosis (Betts et al., 1968).

In an effort to determine the mechanisms involved in sulfatide accumulation, we undertook a targeted analysis of ceramides in the frontal cortex (Fig. 2). Previous evaluations of ceramides reported (Schwarz et al., 2008) unaltered levels in gray matter and augmented levels in white matter in the frontal cortex in schizophrenia. Our data for gray matter consistently demonstrated decreased ceramide levels and parallel increases in sulfatides and lactosylceramides, suggesting that ceramide metabolism via the galactosylceramide and glucosylceramide pathways respectively, is augmented in schizophrenia. This is further supported by the lack of alterations in hydroxyceramides, phytoceramides, ethanolamine ceramides, and sphingomyelins (Fig. 2) in the frontal cortex in schizophrenia (this study; Pearce et al., 2009).

These data support altered sphingolipid metabolism and function in the frontal cortex in schizophrenia. In addition we have previously reported that NAPS, potential biomarkers of neuronal stress, are selectively elevated in schizophrenia (Wood, 2014; Wood and Holderman, 2015). These observations were validated in this study of a third cohort of frontal cortex samples. In addition, we also performed a targeted analysis of NAPEs and NAPEp. For the first time, we report that these biomarkers of neuronal stress (Hansen et al., 1997; Hansen et al., 2000; Hansen et al., 2001; Guan et al., 2006; Kilaru et al., 2011; Janfelt et al., 2012; Luptakova et al., 2018) are elevated in the frontal cortex in schizophrenia. Increases in the levels of these acylated structural glycerophospholipids were paralleled by decrements in the levels of NAEs, the endocannabinoid metabolites of NAPEs, as previously reported (Muguruza et al., 2013). However, the complex roles of endocannabinoids in the etiology of positive and negative symptoms, or cognitive

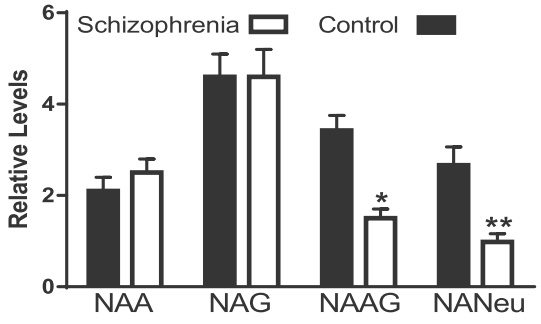

Fig. 6. Relative levels of *N*-acetyl amino acids and sialic acid in the frontal cortex in schizophrenia. NAA, *N*-acetylaspartate; NAG, *N*-acetylglutamate; NAAG, *N*-acetylaspartylglutamate; NANeu, *N*-acetylneuraminic acid (sialic acid). *, p 0.018; p 0.0025.

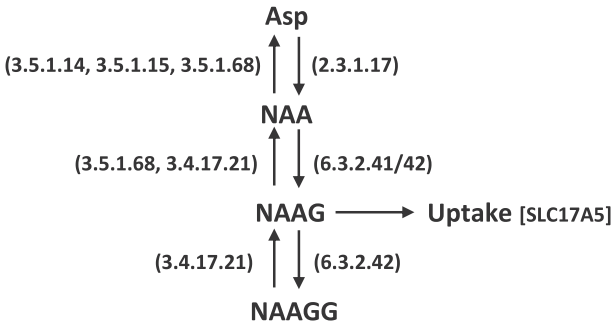

Fig. 7. Schematic presentation of *N*-acetylaspartylglutamate (NAAG) biosynthesis, uptake, and metabolism. Asp, aspartate; NAA, *N*-acetylaspartate; NAAGG, *N*-acetylaspartylglutamyl-glutamate. SLC17A5 (sialin, H^+ nitrite and H^+ sialic cotransporter); 2.3.1.17 (Asp-*N*-acetyltransferase); 6.3.2.41 (NAAG/NAAGG synthase); 6.3.2.42 (NAAG/NAAGG synthase); 3.5.1.14 (aminoacylase I); 3.5.1.15 (aspartoacylase); 3.5.1.68 (*N*-formylglutamate deformylase); 3.4.17.21 (glutamate carboxypeptidase II; NAAG peptidase).

dysfunction, in schizophrenia remains to be more clearly delineated (Leweke et al., 2018).

In addition to decrements in NAE levels, we also monitored a specific decrease in the relative levels of *N*-arachidonyltaurine (NAT 20:4). *N*-Acyl taurines, which are potent modulators of TRPV1 and TRPV4 calcium channels, are synthesized in peroxisomes via acyl-CoA:amino acid *N*-acyltransferase-like 1 (ACNAT1) (Reilly et al., 2007) and like NAEs are metabolized by FAAH (Saghatelian et al., 2006). Oxidative metabolism of *N*-arachidonoyl taurine (NAT 20:4) also generates the inflammatory leukotriene, 12-hydroperoxy eicosatetraenoic acid (12-HETE; Turman et al., 2008). Our data are the first demonstration of NAT 20:4 in human brain and the first report of decreased levels in the frontal cortex in schizophrenia. The impact of this deficit on brain calcium channel function in schizophrenia remains to be defined.

The observed alterations in structural lipid biomarkers of neuronal stress in the cortex may be consistent with speculations regarding the role(s) of abnormal cortical glutamate neurotransmission in schizophrenia (Howes et al., 2015; Uno and Coyle, 2019). In this regard, decrements in NAAG in the frontal cortex in schizophrenia have been reported with post-mortem studies (Zhang et al., 2016) and in vivo with disease progression, utilizing ^{1}H-MRS imaging (Rowland et al., 2013; Liemburg et al., 2016). We also monitored lower levels of this acetylated dipeptide in post-mortem frontal cortex from patients with schizophrenia. NAAG is synthesized from *N*-acetylaspartate (NAA; Fig. 7) by NAAG synthase (Becker et al., 2010) and acts to decrease glutamate release via agonism at metabotropic glutamate 3 (mGluR3) receptors (Ghose et al., 2009a; Ghose et al., 2009b; Neale and Olszewski, 2019). Inactivation of NAAG (Fig. 7) involves uptake via sialin (SLC17A5; Lodder-Gadaczek et al., 2013), metabolism by NAAG peptidase (Olszewski et al., 2004; Olszewski et al., 2012; Olszewski et al., 2017; Ghose et al., 2009a; Takatsu et al., 2011; Janczura et al., 2013), and/or metabolism to *N*-acetylaspartylglutamylglutamate (NAAGG) by NAAG synthase II (Lodder-Gadaczek et al., 2011). Of significant clinical interest to understanding the pathophysiology of schizophrenia, are the observations that NAAG peptidase inhibitors enhance cognitive function (Janczura et al., 2013) and block the cognitive deficits induced by the NMDA antagonist dizocilpine (Olszewski et al., 2012). These data suggest that there may be a link between the observed decrements in cortical NAAG and cognitive deficits which are universal in patients with schizophrenia. As a result of these data, clinical evaluation of NAAG peptidase inhibitors as cognitive enhancers in schizophrenia (Rais et al., 2015) and multiple sclerosis (Rahn et al., 2012) has been suggested.

In addition to decrements in cortical NAAG levels, we monitored similar decreases in the levels of *N*-acetylneuraminic acid (sialic

acid) another substrate for the sialin transporter. Decreases in the levels of *N*-acetylneuraminic acid in platelets from patients with schizophrenia has been reported previously (Sirota et al., 1988). Of interest to neuronal function, decreased availability of *N*-acetylneuraminic acid may be responsible for the decreased levels of polysialic acid-neural cell adhesion molecule complexes (PS-NCAM) in interneurons of the prefrontal cortex in schizophrenia (Gilabert-Juan et al., 2012). These data are relevant to schizophrenia in that PS-NCAM complexes are involved in neuronal connectivity and specifically in spatial learning (Becker et al., 1996). Such findings are extremely complex since *N*-acetylneuraminic acid is both a key component of PS-NCAM complexes and is a major constituent of structural glycosphingolipids (Tettamanti et al., 2003; Schnaar, Gerardy-Schahn, Hildebrandt., 2014), generated by the glucosylceramide limb of sphingolipid metabolic pathways (Fig. 2). As is the case with sulfatides (Boggs et al., 2010; Boggs, 2014; Wood et al., 2014; Wood and Holderman, 2015), these glycosphingolipids (Schnaar et al., 2014) are essential components of glycosynapses in the brain.

In summary, our metabolomics and lipidomics studies demonstrate that in schizophrenia, the frontal cortex expresses abnormal metabolism of structural sphingolipids and NAAG, a neuromodulator of excitatory amino acid transmission. These data, along with elevations in NAPS and NAPES, biomarkers of neuronal stress, suggest that multiple cortical neuronal pathways are likely to be affected in schizophrenia. Our data demonstrating decrements in *N*-acetylneuraminic acid and increases in lactosyl ceramides also strongly support the need for a detailed analysis of gangliosides in future studies of the frontal cortex in schizophrenia.

Acknowledgements

Human tissues were obtained from the NIH Neurobiobanks: Brain and Tissue Bank at the University of Maryland, Baltimore, MD; Brain Endowment Bank at the University of Miami, Miami, FL; and the Human Brain and Spinal Fluid Resource Center at the University of California, Los Angeles, CA. This work was funded by the College of Veterinary Medicine, Lincoln Memorial University.

Declaration of competing interest

The author declares no competing financial interests. The funding source also had no role in these studies.

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Role of funding source

None.

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